Polyamine Metabolism in MRC-5 Cells Infected with Human Cytomegalovirus

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SUMMARY

The rate of putrescine uptake into MRC-5 cells increased markedly following infection with human cytomegalovirus (HCMV). Enhanced incorporation occurred immediately after infection and the highest levels were attained following the production of infectious, progeny virus. Parallel kinetic changes in the utilization of radio-labelled putrescine were shown by the amounts of spermidine and spermine recovered from infected cells as radioactive derivatives. A temporal correlation was found between these changes in polyamine metabolism and the synthesis of virus DNA. Methylglyoxalbis(guanlyhydrizone), an inhibitor of spermidine and spermine synthesis, did not affect virus replication if HCMV-infected cells were exposed to the inhibitor after completion of the eclipse phase of the virus growth cycle. These results show that polyamine metabolism is required only during the initial stages of HCMV replication.

INTRODUCTION

The polyamines spermidine and spermine have been shown to be present in purified preparations of several DNA-containing viruses including vaccinia virus (Lanzer & Holowczak, 1975), herpes simplex virus (Gibson & Roizman, 1971) and adenovirus (Shortridge & Stevens, 1973). Their presence suggests that synthesis of polyamines within the host cell is required for the replication of these viruses. Ornithine decarboxylase (EC 4.1.1.17) initiates the metabolic pathway leading to polyamine synthesis in many animal tissues (Pegg, 1970) and ornithine may be derived from arginine by hydrolysis with arginase (EC 3.5.3.1). Therefore, the requirement shown for arginine in the replication of vaccinia virus (Holtermann, 1969), herpes simplex virus (Tankersley, 1964) and adenovirus (Rouse & Schlesinger, 1967) may be related, in part, to a need for polyamine synthesis.

Human cytomegalovirus (HCMV), another member of the herpesvirus group, is a species-specific pathogen characteristically associated with congenital defects following intra-uterine infection, although latent infections also occur, and it is the most important known infectious cause of mental retardation (Stern, 1975). The replication of HCMV in human diploid fibroblast cells is dependent upon the availability of arginine (Garnett, 1975). A recent study has described a marked stimulation of ornithine decarboxylase activity following HCMV infection of low serum-arrested, confluent monolayers of whole human embryo cells (Isom, 1979). In another report (Tyms et al. 1979) inhibitors of polyamine metabolism have been shown as potent inhibitors of the growth of HCMV in MRC-5 cells. Thus, these observations suggest that continued polyamine biosynthesis is required for HCMV replication.
The present study describes the changes that occur in polyamine metabolism in MRC-5 cells following HCMV infection. These changes are related temporally to the production of infectious, progeny virus and it is shown that continued polyamine metabolism is essential only during the early events of the replication cycle.

**METHODS**

**Cells.** MRC-5 cells, a human diploid cell line (Jacobs et al. 1970), were obtained from Centre for Applied Microbiology and Research, Porton Down, Wilts. Monolayer cultures were prepared in 100 cm² glass bottles for growth of virus stocks, in 45 cm² glass bottles for experiments and in 16 mm diam. wells in microtitration plates (Flow Laboratories, Irvine, Scotland) for titration of virus. Growth medium consisted of Eagle's minimum essential medium supplemented with 10% foetal calf serum and confluent monolayers were maintained in the same defined medium containing 2% foetal calf serum. All cultures were grown and maintained at 37 °C.

**Virus.** Virus stocks were prepared by infecting confluent monolayer cultures of MRC-5 cells with HCMV, the Rawles strain, using 0·5 to 1 p.f.u./cell. This virus strain was kindly supplied by Professor H. Stern. From 6 to 10 days p.i. extracellular virus was recovered by replenishing the maintenance medium at daily intervals. The infected medium containing up to $10^7$ p.f.u./ml was clarified by centrifugation at 500 g for 10 min and stored in liquid nitrogen.

**Procedure for infection.** In all experiments confluent cell monolayers were infected by exposure for 2 h at 37 °C to virus inocula calculated to give an input multiplicity of 5 p.f.u./cell. At the end of the adsorption period the inoculum was replaced with appropriate maintenance medium.

**Virus assay.** Infectivity titrations were made initially using a modified fluorescing cell method (Tyms, 1978) and confirmed subsequently by plaque formation using monolayers of MRC-5 cells maintained under 0·5% agarose overlay. At approx. 8 days p.i. plaques were visualized by staining with methylene blue.

**Polyamine analysis.** Uninfected or HCMV-infected cells were resuspended using 0·001% Pronase, washed with PBS and a sample removed for protein estimations made with the Folin phenol reagent (Lowry et al. 1951). The remaining cells (approx. $5 \times 10^6$ cells/sample) were sedimented by centrifugation at 500 g for 5 min and resuspended in 0·5 ml 0·2 M perchloric acid in 2% SDS for extraction of polyamines. Dansyl derivatives were prepared and analysed essentially by the method of Seiler (1970). Briefly, 0·2 ml samples of the acid-soluble extracts were mixed with 50 mg sodium carbonate and 0·4 ml freshly prepared dansyl chloride (Sigma, Poole, Dorset) in acetone (30 mg/ml) was added. The reaction was allowed to proceed at room temperature in the dark for 16 h after which time 0·1 ml 10% (w/v) proline was added to remove excess dansyl chloride. Dansylated polyamines were extracted with 0·5 ml benzene by vigorous shaking and the organic phase concentrated for extraction of polyamines. Dansyl derivatives were prepared and analysed essentially by the method of Seiler (1970). Briefly, 0·2 ml samples of the acid-soluble extracts were mixed with 50 mg sodium carbonate and 0·4 ml freshly prepared dansyl chloride (Sigma, Poole, Dorset) in acetone (30 mg/ml) was added. The reaction was allowed to proceed at room temperature in the dark for 16 h after which time 0·1 ml 10% (w/v) proline was added to remove excess dansyl chloride. Dansylated polyamines were extracted with 0·5 ml benzene by vigorous shaking and the organic phase concentrated by evaporation to dryness in vacuo followed by reconstitution in 50 μl benzene. The concentrated material was applied in 30 μl amounts to 0·25 mm silica gel G plates (Anachem Ltd., Luton, Beds.) and the plates developed with ethyl acetate:cyclohexane (2:3, v/v). After chromatography the separated derivatives were detected either by fluorescence or by autoradiography, identified by reference to appropriate standards and radio-labelled material recovered by elution into a liquid scintillation system (3·5 g PPO, 50 mg POPOP/l in Metapol:toluene, 1:2, v/v). Radioactivity was measured in a Packard liquid scintillation spectrometer.

**Chemicals.** Radio-labelled putrescine 1,4-14C-dihydrochloride (116 mCi/mmol), spermidine-14C-trihydrochloride (122 mCi/mmol) and spermine-14C-tetrahydrochloride (120 mCi/
Polyamine metabolism in HCMV-infected cells

RESULTS

Growth curve of HCMV in MRC-5 cells

To establish the appropriate, temporal parameters for the investigation of polyamine metabolism, initial experiments were made to determine the kinetics of HCMV replication in MRC-5 cells. One-step growth conditions were achieved by exposure to an inoculum equivalent to 5 p.f.u./cell and infectivity titres of cell-associated or extracellular virus were determined at 12 h intervals until 216 h p.i. (Fig. 1). The results obtained show that within 12 h p.i. the titre of input virus had declined to undetectable levels and this eclipse phase was maintained until 48 h p.i. From this time infectivity titres of cell-associated virus increased until maximum titres were attained at 96 h p.i. and maintained until 192 h p.i. Extracellular progeny virus was detected initially at 72 h p.i. and at 120 h p.i. had reached maximum titres which subsequently paralleled the level of cell-associated virus.

Putrescine uptake into uninfected and HCMV-infected MRC-5 cells

On the basis of the temporal characteristics of HCMV replication in MRC-5 cells, the rate of uptake of labelled putrescine into uninfected and virus-infected cultures was determined by pulse-labelling at consecutive 24 h periods for a total of 144 h. In these experiments one series of cell cultures was infected with HCMV by the methods described. After 2 h adsorption the inocula were removed, replaced with maintenance medium and 14C-putrescine (0.1 μCi/ml) added to duplicate, infected cultures either immediately or at successive 24 h intervals. The same procedure was used throughout with a second series of uninfected cultures except that during the adsorption period the control cultures were exposed to a similar volume of maintenance medium. Appropriate uninfected and HCMV-
infected cells were recovered at the end of each 24 h period and the amount of acid-soluble radioactivity determined.

The rate of incorporation of radioactivity available as 14C-putrescine into uninfected MRC-5 cells showed two different patterns in the initial stages. These differences appeared to be related to the population density of the cell cultures at the start of the experiment and to the particular batch of foetal calf serum used in the maintenance medium. If the cell cultures were used immediately after the monolayers had reached confluence, as assessed by microscopic examination, there was an increased rate of incorporation during the first 24 h period. After this early stimulation the rate of incorporation into uninfected cultures declined to a lower level which was maintained from 48 h until the end of the experiment. In experiments initiated several days after confluent monolayers had been formed, the rate of incorporation was relatively constant during each successive 24 h period (Fig. 2).

The amount of radioactivity present in acid-soluble extracts of HCMV-infected cells at 24 h p.i. was enhanced markedly compared with either type of control culture (Fig. 2). Subsequent pulse-labelling periods showed increased rates of incorporation of labelled putrescine into infected cells to reach the highest levels at 72 to 96 h p.i. At these times the rate of incorporation of radioactivity into virus-infected cells was up to 20-fold greater than that for uninfected cells. This difference is halved if the amount of radioactivity measured is related to cell protein due to the increased volume of HCMV-infected cells which occurs at an early stage in the replication cycle. The rate of incorporation declined subsequently to minimal levels at 144 h p.i. There was a 20% reduction in the amount of radioactivity in the maintenance medium at the end of the pulse-labelling period associated with the highest rate of incorporation. In all experiments similar amounts of acid-soluble, cellular radioactivity were extracted with the SDS-perchloric acid mixture or with 10% trichloroacetic acid under conditions described previously (Archard & Williamson, 1971). Using the latter method less than 0.1% of the total radioactivity in either uninfected or HCMV-infected cells was recovered as acid-precipitable materials.

Synthesis of spermidine and spermine in uninfected and HCMV-infected MRC-5 cells

Following the demonstration of enhanced levels of acid-soluble radioactivity in HCMV-infected cells maintained in the presence of 14C-putrescine, duplicate perchloric acid–SDS extracts were dansylated at the end of each 24 h pulse-labelling period for a total of 144 h by methods described previously. The dansyl-polyamines were extracted into benzene and separated by thin-layer chromatography. Areas of fluorescence and radioactivity detected under u.v. light or autoradiographically, respectively, were identified as dansylated putrescine, spermidine or spermine by reference to appropriate standards. In all samples more than 90% of the radioactivity in acid extracts of whole cells was recovered in the benzene extracts as dansylated derivatives.

In uninfected MRC-5 cell cultures showing a relatively constant rate of incorporation of 14C-putrescine into acid-soluble material at all pulse-labelling periods, a similar pattern of radioactivity associated with putrescine, spermidine and spermine was observed (Fig. 3). In HCMV-infected cells, however, increased amounts of radio-labelled putrescine were found from 24 h p.i. and further increases occurred in successive pulse-labelling periods until maximum levels of radioactivity were attained at 96 h p.i. At later times the amounts of radioactivity associated with putrescine declined. These changes were paralleled by the amounts of radioactivity in virus-infected cells recovered as spermidine and spermine (Fig. 3). The results show that putrescine serves as a precursor for spermidine and spermine synthesis during HCMV replication. At the maximum rate of utilization of 14C-putrescine these polyamines were recovered from virus-infected cells as radio-labelled derivatives with an activity 20-fold greater than control, uninfected cells. Thus, the synthesis of spermidine
Polyamine metabolism in HCMV-infected cells

Fig. 3. Rates of synthesis of radio-labelled spermidine and spermine from $^{14}$C-putrescine in uninfected and HCMV-infected MRC-5 cells. O—O, Putrescine; □—□, spermidine; and △—△, spermine extracted from uninfected cells: ●—●, putrescine; ■—■, spermidine; and ▲—▲, spermine extracted from HCMV-infected cells.

Fig. 4. DNA synthesis in uninfected (○—○) and HCMV-infected (●—●) MRC-5 cells.

and spermine from putrescine under these conditions is enhanced markedly in MRC-5 cells following HCMV infection.

**DNA synthesis in HCMV-infected MRC-5 cells**

In order to relate the changes in polyamine metabolism to other events in the virus replication cycle, the kinetics of DNA synthesis were determined in HCMV-infected cells. Cultures of MRC-5 cells were used for these experiments immediately after confluent monolayers had been established. Infected cultures were pulse-labelled with $^{3}$H-thymidine (0.5 μCi/ml) for 2 h periods at various times after infection and the radioactivity associated with labelled DNA was determined as described previously (Archard & Williamson, 1970). Apart from replacement of the virus inocula with an equal volume of maintenance medium, uninfected cultures were subject to the same procedure.

There was a marked increase in the rate of incorporation of $^{3}$H-thymidine into HCMV-infected cells within 6 h of initial exposure to virus; a similar stimulation was seen at this time with uninfected cells (Fig. 4). The rate of incorporation declined rapidly during subsequent pulse-labelling periods until a further increase at 24 h which was significantly greater in uninfected than in virus-infected cells. After this time uninfected cells incorporated label at a reduced rate which remained relatively constant. However, higher rates of incorporation into infected cells were observed from 36 h p.i. and maximum levels occurred between 48 h and 96 h p.i. This period of increased DNA synthesis peculiar to HCMV-infected cells is accompanied by the appearance of intranuclear inclusions containing virus-specific DNA (McAllister et al. 1963; Huang et al. 1973). These results suggest, therefore, that there are concomitant increases in polyamine metabolism and in the synthesis of virus DNA during HCMV replication.

**Effect of delayed addition of MGBG on production of infectious virus**

The synthesis of spermidine and spermine in mammalian cells is catalysed by S-adenosylmethionine decarboxylase (EC 4.1.1.50) and methylglyoxalbis(guanylhydrazone) (MGBG)
is a potent inhibitor of this activity (Williams-Ashman & Schenone, 1972). Both polyamine synthesis and HCMV replication in MRC-5 cells are inhibited in the presence of MGBG (Tyms et al. 1979). In the present study infected cultures were transferred to medium containing 0.1 mM-MGBG at various times after infection and yields of infectious virus were determined at 144 h p.i. (Table 1). Exposure of infected cells to maintenance medium containing the inhibitor at any time before 6 h p.i. resulted in a complete inhibition of virus yield. Addition of MGBG at progressively later times resulted in an increased virus yield until at 72 h p.i. infectivity titres determined subsequently were comparable with non-inhibited, infected cultures. In these experiments MGBG was used at a threefold greater concentration than that required to inhibit HCMV replication when added immediately after infection; higher concentrations produced overt cytotoxic effects in uninfected cells. Infected cultures maintained in the presence of 0.1 mM-MGBG until 144 h p.i. but maintained subsequently in the absence of the inhibitor produced yields of infectious progeny virus at later times which were comparable with appropriate controls. These results show a reversible inhibitory effect of MGBG at an early stage in the HCMV replication cycle.

DISCUSSION

Previous studies have reported various changes in polyamine metabolism in cell cultures infected with different DNA-containing viruses. A marked increase in ornithine decarboxylase activity leading to the formation of putrescine, the initial step in polyamine biosynthesis, occurs following infection with HCMV (Isom, 1979). A biphasic increase in the activities of both ornithine decarboxylase and S-adenosylmethionine decarboxylase, together with the levels of putrescine, spermidine and spermine, is caused by polyoma virus infection (Goldstein et al. 1976). In vaccinia virus-infected cells the conversion of ornithine to polyamines continues in spite of a general inhibition of host protein synthesis (Lanzer & Holowczak, 1975) and vaccinia infection results in qualitative changes in ornithine decarboxylase activity (Hodgson & Williamson, 1975). Conversely, this activity appears to be inhibited during the replication of herpes simplex virus type 1 since the conversion of ornithine to putrescine ceases (Gibson & Roizman, 1973) and is accompanied by an inhibition of the synthesis of spermidine and spermine from putrescine (McCormick & Newton, 1975). A similar effect on ornithine decarboxylase activity follows herpes simplex virus type 2 infection (Isom, 1979).

The present investigation has shown that there is a marked stimulation of the uptake of $^{14}$C-putrescine and the synthesis of labelled spermidine and spermine in MRC-5 cells following HCMV infection. These changes, which occurred immediately after infection,
continued during the eclipse phase and the period of increased production of progeny virus in the virus replication cycle. Even in those experiments showing an early, transient increase, the levels of activity in uninfected cultures at all times were significantly lower. Therefore, marked kinetic differences in polyamine metabolism exist between uninfected and HCMV-infected cells. Spermidine and spermine synthesis from putrescine in MRC-5 cells has been shown to be inhibited after infection with either herpes simplex type 1 virus or herpes simplex type 2 virus (Tyms et al. 1979). These different effects suggest a specific, virus-mediated induction of putrescine utilization during HCMV replication.

Elevated ornithine decarboxylase activity accompanied by accumulation of spermidine and spermine has been demonstrated in many model systems of stimulated growth and polyamines have been shown to affect DNA, RNA and protein synthesis in cell-free systems. HCMV replication in human diploid fibroblasts is characteristically associated with enhanced host cell macromolecular synthesis. Both mitochondrial (Furukawa et al. 1976) and cellular (St Jeor et al. 1974) DNA synthesis are stimulated during the first 24 h p.i. but virus-specific DNA is not detected until 48 h p.i. when further stimulation of host-specific DNA synthesis occurs (St Jeor & Hutt, 1977). A similar temporal pattern of thymidine incorporation in MRC-5 cells following HCMV infection was observed in the present investigation with an initial peak at 24 h p.i. coincident with increased incorporation into uninfected cells. A further increase from 36 h p.i. was maintained until 96 h p.i. and at this time the virus DNA which is present in intranuclear inclusions can be visualized by acridine orange staining of HCMV-infected cells (Tyms et al. 1979). These later increases in the rate of thymidine incorporation are coincident with the maximum rates of utilization of labelled putrescine suggesting a correlation between polyamine metabolism and DNA replication.

The cellular levels of spermidine and spermine have been shown to increase markedly in lymphocytes induced to transform by concanavalin A but activation in the presence of MGBG inhibits DNA synthesis whereas RNA and protein synthesis proceed normally (Fillingame et al. 1975). A similar inhibitory effect results from the depletion of spermidine concentrations in rat hepatoma cells following exposure to α-methylornithine (Mamont et al. 1976). Inhibition of HCMV replication by either MGBG or α-methylornithine has been shown previously to be accompanied by a marked reduction in the formation of intranuclear, DNA-containing inclusions suggesting that virus-specific DNA replication is dependent upon continued polyamine metabolism (Tyms et al. 1979). In the present study the effect of delayed addition of MGBG on the subsequent yield of virus shows that only during the eclipse phase of the replication cycle, a period associated temporally with increased DNA synthesis, is there a requirement for polyamine metabolism. This is consistent with the early increase in ornithine decarboxylase activity that occurs in Flow 5000 cells following HCMV infection (Isom, 1979), but apparently inconsistent with the production of infectious, progeny virus in HCMV-infected Flow 5000 cultures maintained in the presence of concentrations of α-methylornithine which inhibit the enzyme activity (Isom & Pegg, 1979). However, although the replication of herpes simplex type 1 virus is insensitive to inhibitors of polyamine metabolism (McCormick & Newton, 1975; Tyms et al. 1979), cell-free synthesis of virus DNA is optimal in the presence of polyamines at concentrations close to their cellular levels (Francke, 1978). Utilization of host cell polyamines in DNA synthesis may occur also during polyoma virus replication (Goldstein et al. 1976) but de novo synthesis is required for the association of virus DNA with cytoplasmic inclusions in vaccinia virus-infected cells (Williamson 1976). Thus, there is considerable evidence which indicates a requirement for polyamines in both cellular and virus DNA synthesis. Although the precise function is not known, the initiation of new
replication units appears influenced by spermidine and spermine (Krokan & Eriksen, 1977). Such a role is consistent with the essential requirement for polyamine synthesis during the early stages of HCMV replication demonstrated in the present study.

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REFERENCES


Polyamine metabolism in HCMV-infected cells


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